Inhibition of Human Epidermal Transglutaminases In Vitro and In Vivo by Tyrosinamidomethyl Dihydrohaloisoxazoles

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Tyrosinamidomethyl dihydrohaloisoxazoles (THX) irreversibly inhibit isolated epidermal transglutaminases and ionophore-induced cell envelope formation in malignant human keratinocytes. In cultured human foreskin keratinocytes cultured in 10^{-5} M THX for 5 days, soluble and particulate transglutaminases were inhibited by 90% and 44–51%, respectively. Spontaneous cell envelope formation was inhibited up to 54%. When THX-treated keratinocytes were simultaneously incubated with 10^{-5} M retinoic acid (RA), there was enhanced inhibition of cell envelope formation compared to either agent alone. The inhibitors were equally effective in keratinocytes incubated with fetal calf serum or delipidized serum. After THX was applied to normal human thoracic skin in vivo for 9 d, the soluble and particulate transglutaminases isolated from suction blister epidermis were inhibited 30% and 40%, respectively. THX may be effective in inhibiting both soluble and particulate transglutaminase activity in disorders with increased transglutaminase activity. *J Invest Dermatol 97:156–158, 1991*

In epidermis, transglutaminases catalyze the formation of ϵ-(γ-glutamyl) lysine bonds between proteins during the normal formation of cell envelopes in the stratum corneum [1,2]. Recently, a new set of compounds, THX, which function as specific irreversible transglutaminase inhibitors, have been synthesized [3,4]. These compounds have been shown to be effective inhibitors of ionophore-induced envelope formation in SCC-9, a neoplastic human cell line [4]. We now report the ability of these compounds to inhibit transglutaminases in vitro in normal human cultured keratinocytes and in vivo in normal human epidermis. Furthermore, we show that this inhibition in normal human keratinocytes is associated with a reduction in the spontaneous formation of cornified envelopes.

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Abbreviations:

BSA: bovine serum albumin
DMSO: dimethyl sulfoxide
DTT: DL-dithiothreitol
FCS: fetal calf serum
PBS-A: phosphate-buffered saline-A
RA: retinoic acid
SDS: sodium dodecyl sulfate
TCA: trichloroacetic acid
THX: tyrosinamidomethyl dihydrohaloisoxazoles

MATERIALS AND METHODS

Reagents Transglutaminase inhibitors were synthesized by Syntex Research (Canada). The compounds tested are tyrosinamidomethyl dihydrohaloisoxazoles with different halide groups and different chiralities as listed: RS-31819 (no halide, 5*S* and 5*R*); RS-10823 (Br, 5*R*); RS-10025 (Cl, 5*S*); RS 10495 (Br, 5*R*). Retinoic acid (RA), bovine serum albumin (BSA), phenylmethylsulfonylfluoride (PMSF), DL-dithiothreitol (DTT), and dimethyl sulfoxide (DMSO) were from Sigma Chemical Co. (St. Louis, MO). ^{14}C-putrescine (specific activity 101 mCi/mmol) was obtained from Amersham International Corp. (Arlington Heights, IL). Other reagents and solvents were of the highest grades available. Delipidized serum (a gift of Victor Stevens) was prepared according to published techniques [5].

Tissue Culture Studies Human neonatal foreskin keratinocytes were cultured on an irradiated 3T3 feeder layer following the techniques of Rheinwald and Green [6]. In each experiment, cells from one individual were used. Each 60-mm Corning culture dish was seeded with about 1×10^5 cells and grown in supplemented DMEM (Hazleton; Lenexa, KS) with 20% (V/V) FCS (Hyclone; Logan, UT). The cells were cultured at 37°C in 5% CO_2 for 5–7 d until about 50% confluent. Inhibitors were dissolved in 100% DMSO and added daily to fresh media to a final concentration of 0.1% DMSO. Experiments were stopped when plates were 90–95% confluent.

Total Protein Extraction and Cell Envelope Determination Cells were extracted with 0.5 ml of 20 mM DTT in 20% (W/V) SDS and incubated at 37°C for 20 min. After centrifugation the cell pellets were resuspended, and four aliquots were counted microscopically. The number of envelopes was expressed as envelopes per culture dish.

Transglutaminase Activity in Cultured Human Keratinocytes Culture dishes were rinsed twice with 5 ml of PBS-A. Each dish was then scraped with two 500-μl aliquots of 10 mM Tris

HCl, 10 mM DTT, 0.5 mM EDTA, and 5 μg/ml PMSF adjusted to a final pH of 7.4. The two aliquots from each dish were combined and sonicated on ice for 10 sec 5–10 times. The mixtures were then centrifuged at 17,000 × *g* for 15 min at 4°C. The supernatant was collected, and the pellet was suspended in the starting volume with the same extraction buffer. All samples were then stored at −70°C until assayed. The protein was determined in triplicate using a modified Lowry technique [7] with BSA as a standard. Transglutaminase activity in sample aliquots was determined by the technique of Lichti et al [8] with minor modifications, and radioactivity was measured in a Packard Tricarb Scintillation Counter.

Transglutaminase Activity in Human Suction Blister Epidermis

Volunteers and Treatment: Two experiments were performed using nine informed subjects in each. Subjects had normal skin and consisted of nine women (average age 53 years) and nine men (average age 37 years). For each subject four treatment sites of 25 cm^2 were delineated on the lower abdomen. Two sites were placed on each side of the midline. A gel containing 2.5% RS-10025 was used as the test formulation. Control gel lacked active drug. Active gel was applied to either the right or left treatment sites, and gel vehicle alone was applied to the contralateral side for each subject. Thus, each subject served as his or her own control. A volume of 0.15 ml gel was applied to each site 3 times daily for 9 d and once on the morning of day 10 (total of 28 applications).

Blister Harvest: On the afternoon of day 10, immediately prior to blistering, each site was swabbed with acetone to remove any nonabsorbed formulation. Three blisters were then raised on each site using a custom designed Plexiglass vacuum manifold with 5-mm diameter chambers and a negative pressure of 300–400 mm Hg generated with a vacuum pump. The vacuum was released when a blister filled the 5-mm chamber. Blister tops were then swabbed with ethanol and excised with a scissors. Excised tops were then washed in sterile saline and then in acetone. In our preliminary experiments this technique was found to yield full-thickness epidermis.

Transglutaminase Activity: For measurements of transglutaminase activity in suction blister epidermis, each determination was made from the six pooled blisters on each treatment side of a subject. Thus, each subject yielded one data point each for control and active treatments. Pooled blisters were frozen in homogenizing buffer containing 0.25 M sucrose, 50 mM Tris-HCl pH 7.5, and 1 mM EDTA. After thawing in cold water, samples were homogenized at 0°C for 1 min using a motor-driven glass/glass homogenizer. The homogenate was centrifuged at 12,000 × *g* for 15 min at 4°C. The supernatant and two subsequent pellet washes were pooled and designated the "soluble" fraction. The pellet was resuspended in homogenizing buffer containing 1% (V/V) Triton-X 100 and 20 mM DTT, incubated at 37°C for 5 min and centrifuged at 12,000 × *g* for 15 min at 4°C. The supernatant from this step was designated the "particulate" fraction.

Transglutaminase activity was assayed by adding an aliquot of sample to 1.0 ml of a 50 mM Tris-HCl pH 8.1 assay mix containing 10 mM $CaCl_2$, 5 mM DTT, 1.2 mg dimethylcasein, and 1 mM ^{3}H-putrescine (sp. act. 10 mCi/mmol). Tubes were incubated at 37°C for 60 min in a shaking water bath. The reaction was stopped by adding 0.3 ml of 25% (V/V) TCA containing 0.1% (W/V) unlabeled putrescine. Tubes were incubated for an additional 30 min at RT and centrifuged at 150 × *g* for 15 min. The supernatant was discarded, and the pellet was washed twice with 0.75 ml of 5% TCA containing 0.1% unlabeled putrescine and once with 0.75 ml of 100% ETOH. The pellet was then digested in 0.5 ml of NCS tissue solubilizer, placed in toluene liquiflour, and the radioactivity determined using a scintillation counter. For background blanks boiled enzyme samples were used. Protein in retained samples of the soluble fraction was determined using the Pierce BCA protein assay (Pierce Chemical Co., Rockford, IL).

Table I. Inhibition of Transglutaminase Activity in Human Foreskin Keratinocytes After Culturing with Transglutaminase Inhibitors[a]

Condition	Inhibition	Transglutaminase Activity nmol/mgm protein/h Supernatant	Pellet
Control		3.41	3.46
Vehicle (DMSO 0.1%)		3.51	3.85
RS-31819	None	2.95 (16)	3.20 (17)
RS-10823	High	6.28 (92)	1.75 (52)
RS-10025	High	0.29 (92)	2.25 (42)
RS-10495	Low	0.54 (85)	2.41 (30)

[a] Each data point is the mean of duplicate determinations. The inhibitors at 10^{-5} M and the media were changed daily and present for 5 d in a final 0.1% DMSO. Degree of inhibition determined from in vitro inhibition studies [3]. The rank order of inhibition with crude human stratum corneum transglutaminase (data not shown) was the same as that determined with cow snout transglutaminase [3]. Percent inhibition in parenthesis compares the activity with DMSO (0.1%) control.

Table II. Inhibition of Cell Envelope Formation by Transglutaminase Inhibitors[a]

Condition	Envelopes/dish ×10^{-3}	% Inhibition[b]
Control	318	
DMSO (0.1%)	296	
RS-31819 (N)	293	1
RS-10823 (H)	137	54
RS-10025 (H)	168	43
RS-10495 (L)	211	29

[a] Data from duplicate dishes. The inhibition labeling is high (H), low (L), or none (N) as determined by in vitro reactivity with epidermal transglutaminase [3]. Cells were grown in transglutaminase inhibitors at 10^{-5} M in a final concentration of 0.1% DMSO.

[b] Percent inhibition compares activity to the 0.1% DMSO control.

RESULTS

Cultured Keratinocytes Human keratinocytes cultured in the presence of 10^{-5} M inhibitor had no changes in keratinocyte morphology such as vacuoles, extended cell processes, or differences in cell size, and there was no evidence of increased desquamation of cells. Soluble and particulate transglutaminases from the normal human keratinocytes were inhibited by the haloisoxazoles (Table I). RS-31819, a structural analogue to the inhibitors without any inhibitory effect on extracted transglutaminases, did not significantly alter transglutaminase specific activity in the soluble or in the particulate fraction. The active inhibitors (RS-10025 and RS-10823) were found to inhibit soluble activity almost 90% and inhibit particulate activity between 44% and 51%.

When keratinocytes were cultured with inhibitors in untreated serum there was a 43% and 54% inhibition of cell envelope formation with the more potent inhibitors RS-10823 and RS-10025, respectively, and 29% with RS-10495 a less potent inhibitor (Table II). The non enzyme-inhibiting analogue, RS 31819, did not inhibit envelope formation (Table II).

Table III. Effects of Retinoic Acid and a Transglutaminase Inhibitor on Cell Envelope Formation[a]

	Normal Serum		Delipidized Serum	
	Envelopes/dish Inhibition[b]		Envelopes/dish Inhibition[b]	
Condition	×10^{-3}	%	×10^{-3}	%
Control	178		280	
DMSO (0.1%)	191		245	
RA	84	56	101	58
RS-10823	108	43	140	43
RS-10823 + RA	40	79	68	72

[a] Cells were grown in transglutaminase inhibitors at 10^{-5} M in a final concentration of 0.1% DMSO.

[b] Percent inhibition compares activity to the 0.1% DMSO control.

Table IV. The Effect of Topical Treatment with RS-10025 on Transglutaminase Activity in Human Suction Blister Epidermis[a]

Treatment	Soluble Protein[b]	Transglutaminase Activity[c]	
		Soluble	Particulate
Gel Vehicle	258 ± 16	2.18 ± 0.23	0.75 ± 0.08
2.5% RS-10025	257 ± 13	1.42 ± 0.15[d]	0.38 ± 0.04[d]

[a] Data is mean ± SE for 18 subjects.
[b] μg protein/sample.
[c] nmoles putrescine bound/sample/h.
[d] $p < 0.0003$ versus vehicle (Student two-sided t test).

In a separate experiment (Table III) RS-10823 inhibited envelope formation by 43% in both delipidized and untreated fetal calf serum. As expected, there were more envelopes per dish in the delipidated serum compared to normal serum for both control and DMSO containing dishes (Table III). Retinoic acid at 10^{-5} M inhibited envelope formation by 56% in normal serum and by 58% in delipidized serum. In both delipidized and untreated serum there was greater inhibition of cell envelope formation when RA and RS-10823 were combined than with either agent alone.

Human Skin In Situ Throughout the study, the gross appearance of drug-treated sites and vehicle-treated sites were the same. Neither treatment produced irritation, and there was no difference in the re-epithelialization time of the harvested sites in the two areas. Furthermore, drug treatment had no effect on epidermal soluble protein levels (Table IV). However, RS-10025 treatment did inhibit the particulate and soluble transglutaminase activity in suction blister epidermis in 14 of the 18 subjects examined (Table IV, Fig 1). The mean inhibitions per subject were 30% for soluble and 40% for particulate enzyme activity. The increase in enzyme activity in two subjects is unexplained.

DISCUSSION

The THX transglutaminase inhibitors blocked the action of soluble and particulate transglutaminases in cultured human keratinocytes and inhibited the spontaneous production of cell envelopes when present continuously during cell culture. The inhibitors that exhibited the highest activity against isolated transglutaminase were also the most potent inhibitors against soluble and particulate enzymes in cultured keratinocytes. In addition, RS-10025, when tested in vivo by topically applying this inhibitor on human skin, significantly decreased both the soluble and particulate transglutaminase activity of human epidermis. Apparent differences between the degree of inhibition of the soluble and particulate transglutaminases will have to be verified on purified forms of the enzymes.

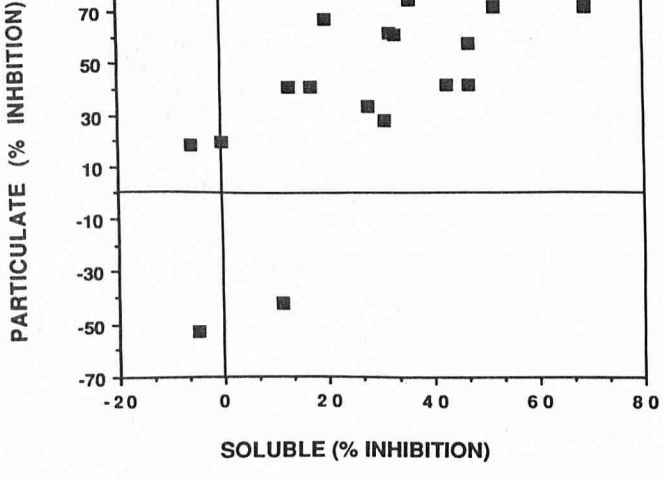

Figure 1. Inhibition of transglutaminase in human suction blister epidermis harvested after topical treatment for 10 d with 2.5% RS 10025 gel. Baseline values were obtained from sites treated with gel lacking drug (see *Materials and Methods*). Each *square* represents one of the 18 subjects tested. Negative values indicate stimulation of activity.

Consistent with the fact that endogenous retinoids suppress spontaneous envelope formation [9–11], we found that cultures grown in delipidized serum that lack vitamin A produced more cell envelopes per dish. Conversely, when RA is added to delipidized serum, cornified envelope formation is suppressed. We observed that the addition of the potent transglutaminase inhibitor RS-10823 to retinoid-containing cultures resulted in a further suppression of envelope formation. Retinoids are thought to inhibit cornified envelope formation by inhibiting synthesis of both particulate epidermal transglutaminase and its substrates [10,12,13], whereas the haloisoxazoles act directly on the enzyme. Thus, we would expect the two agents to have an additive effect, as was observed.

These studies show that haloisoxazoles are inhibitors of soluble and particulate transglutaminases and spontaneous (non-ionophore-induced) cell envelope formation in normal cultured human keratinocytes. These compounds are also able to inhibit the soluble and particulate transglutaminases in normal human skin in vivo. In addition to being important reagents for defining the action of transglutaminases, these agents may have therapeutic applications. We are currently investigating whether the inhibitors will be therapeutic in diseases such as acne, where transglutaminase levels in the sebaceous follicles are abnormally altered [14].

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